Have *p53* Gene Mutations and Protein Expression a Different Biological Significance in Colorectal Cancer?

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p53 alterations are considered the most common genetic events in many types of neoplasms, including colorectal carcinoma (CRC). These alterations include mutations of the gene and/or overexpression of the protein. The aim of our study was to assess whether in 160 patients undergoing resective surgery for primary operable CRC there was an association between p53 mutations and protein overexpression and between these and other biological variables, such as cell DNA content (DNA-ploidy) and S-phase fraction (SPF), and the traditional clinicopathological variables. p53 mutations, identified by PCR-SSCP-sequencing analysis, were found in 68/160 patients (43%) and positive staining for p53 protein, detected with the monoclonal antibody DO-7, was present in 48% (77/160) of the cases, with agreement of 57% (91/160). In particular, a significant association was found between increased p53 expression and genetic alterations localized in the conserved regions of the gene or in the L3 DNA-binding domain and the specific type of mutation. Furthermore, both overexpression of p53 and mutations in the conserved areas of the gene were found more frequently in distal than in proximal CRCs, suggesting that they might be "biologically different diseases." Although p53 mutations in conserved areas were associated with flow cytometric variables, overexpression of p53 and mutations in its L3 domain were only related respectively to DNA-aneuploidy and high SPF. These data may reflect the complex involvement of p53 in the different pathways regulating cell-cycle progression. In conclusion, the combination of the mutational status and immunohistochemistry of p53, and flow cytometric data may provide an important insight into the biological features of CRCs.   J. Cell. Physiol. 191: 237–246, 2002. © 2002 Wiley-Liss, Inc.

p53 alterations are considered the most common genetic events in many types of neoplasms, including colorectal carcinoma (CRC) (Hollstein et al., 1991; Lane, 1994). The *p53* gene codifies for a nuclear phosphoprotein which acts as a transcriptional regulator which prevents proliferation of damaged cells, in some cases inducing them to apoptosis (Ryan et al., 2001).

In most normal tissues, the wild-type p53 protein is constitutively expressed at low levels because of a short half-life (5′–40′ depending on the cell cycle phase in which it is evaluated) (Brown and Pagano, 1997) due to a rapid degradation, but it may accumulate in the cell as a result of several stresses, such as DNA damages,

hypoxia, loss of normal growth and survival signals, acidity, inflammatory processes (Fearon, 1998; Takashi and Nakamura, 2000), which may occur in different physiological or pathological situations, including tumorigenesis.

Although the mechanisms regulating p53 accumulation and function are still to be completely clarified, recent studies have demonstrated that a major inhibitor of p53 is MDM2, which binds to the N-terminal transactivation domain of p53 and targets it for ubiquitin-mediated degradation. As MDM2 is a transcriptional target of p53, increased p53 activity leads to increased expression of its own negative regulator (Lane and Hall, 1997). In response to DNA damages and other stresses, however, either by phosphorylation of N-terminal residues of p53 or by other pathways, binding of MDM2 and ubiquitination are inhibited, with a consequent stabilization and accumulation of p53, mostly in the nucleus. By regulating the expression of a number of genes, p53 then induces cell cycle arrest and/or apoptosis (Ryan et al., 2001).

Mutations and deletions of the *p53* gene may offer an obvious selective growth advantage to the neoplastic cells (Fearon, 1998). About 90% of its mutations are found in a region of 600 base pairs including exons 5–8, which contain the nucleotide sequences preserved during evolution and coding for the aminoacids most important for the p53 DNA binding activity (area II, codons 112–141; area III, codons 171–181; area IV, codons 234–258; area V, codons 271–286) (Figure 1) (Levine et al., 1991). Furthermore, within this region several functional domains have been identified, such as loop L2 (between codons 163 and 195), required for the folding and stabilization of the central part of the protein, and loop L3 (between codons 236 and 251) and the LSH motif (L1 loop-sheet-α-helix, between codons 273 and 286), directly involved in the interaction of the protein with DNA (Cho et al., 1994).

Several studies in different neoplasms have revealed a relationship between the presence of mutations within the *p53* gene and over-expression of its protein product (Dix et al., 1994; Bertorelle et al., 1996). Mutated p53 proteins present in adenomas and carcinomas have been found to be metabolically more stable, with a longer half-life (> 6 h), and therefore present at higher levels within the cell (Costa et al., 1995), mostly accumulated in the nucleus. However, other authors report different grades of discordance (Leahy et al., 1996; Veloso et al., 2000) between p53 mutations and overexpression.

The aim of our study was to assess whether in 160 patients undergoing resective surgery for primary operable CRC there was an association between p53 mutations and protein overexpression and also to assess the association between these and other biological variables, as DNA-ploidy and SPF, and the traditional clinicopathological ones.

MATERIALS AND METHODS
Study design

A prospective study was performed on paired tumor and normal tissue samples collected by the Molecular Oncology Section of the University of Palermo from a consecutive series of 160 patients undergoing potentially radical surgical resection for primary operable CRC at a single institution (Department of Oncology, University of Palermo) from January 1988 to December 1992.

The inclusion criteria used were: (a) electively resected primary CRC, (b) processing of fresh paired normal mucosa-tumor samples within 30 min after tumor removal, (c) available DNA from normal and tumor tissue for biomolecular and flow cytometric analysis.

Briefly, the following exclusion criteria were used: (a) history of previous neoplasms, (b) patients from families with familial adenomatous polyposis or hereditary non-polyposis CRC with a highly penetrant genetic predisposition to CRC, (c) synchronous or metachronous CRC, and (d) chemotherapy or radiation therapy prior to surgery.

The patients of this series comprised 84 females and 76 males with a median age of 66 years (range 31–88). In order to avoid evaluator variability in the patients, all resection specimens and microscopic slides were meticulously examined by two independent pathologists (R.M.T. and V.M.) who were not aware of the original diagnosis and of the results of the molecular analysis.
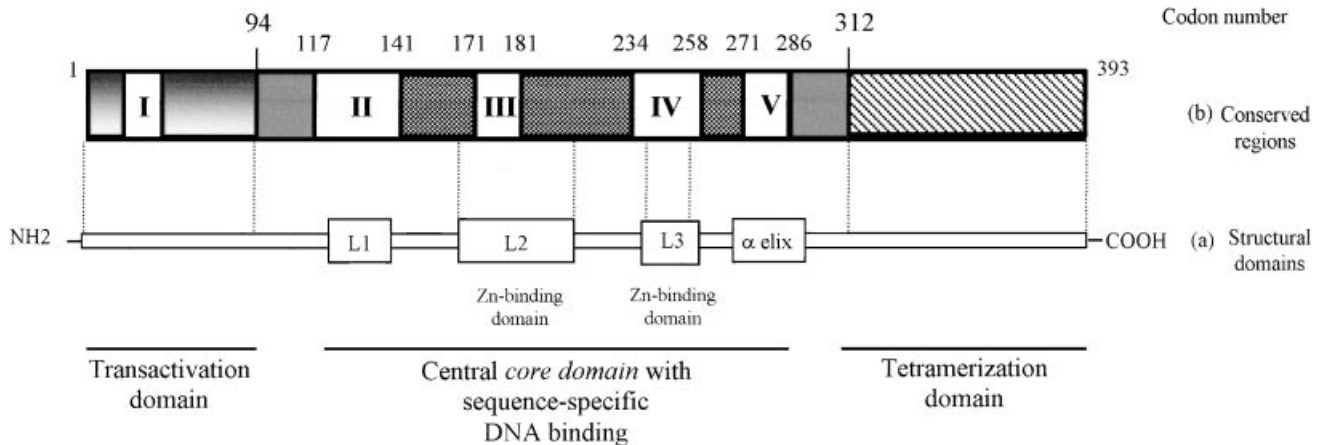

Fig. 1.   Schematic representation of the p53 protein structural domains (**a**) and highly conserved regions of *p53* gene (**b**).

The complete excision of the primary tumor was histologically proven by examination of the resected margins. All tumors were histologically confirmed to be colorectal adenocarcinomas. In addition, the pathologists assessed tumor site (proximal or distal tumors), tumor size, pathological stage according to Turnbull's modification of Dukes' system (Turnbull et al., 1967) (from A to D), presence or absence of lymph node metastases, tumor growth (expansive or infiltrative), tumor grade (histological differentiation), tumor type (NOS or mucinous adenocarcinoma), presence or absence of vascular and lymphatic invasion or tumor lymphocytic infiltrate, type of resection (curative or not curative) (Table 1). Written informed consent was obtained from all patients included in this study. Clinicopathological and follow-up data of all patients have been recorded prospectively in a computerised registry database.

Tissue handling

Multiple samples (6–10) of the primary tumor tissue were taken from different tumor areas (including the core and the invasive edge of the tumor). The portion of primary tumor was obtained by superficial biopsy of either the tumor bulk or the edge of the malignant ulcer for more infiltrative cancer. All tissues were carefully trimmed to remove as much non-neoplastic tissue as possible, avoiding the non-viable areas. Furthermore, multiple samples of normal mucosa (as confirmed by histology) were taken from macroscopically uninvolved area 20–40 cm away from the tumor site, to be used as control for biomolecular and flow cytometric analyses. The tissues were bisected, one half of each sample was processed for pathological examination, and the remaining half of the sample pool was immediately frozen and stored at −80°C until analyzed. The adequacy of the material was checked on frozen tissue sections and only tissue samples with more than 80% tumor content were utilized in subsequent biomolecular and flow-cytometric analyses. Where present, areas with a high content of non-neoplastic cells were removed from the frozen block with a scalpel. Evaluation of each biomolecular

variable (p53 alterations, DNA-ploidy, and S-phase fraction (SPF)) was performed independently by researchers who had no knowledge of the clinical data for the samples.

Detection of *p53* gene mutations

DNA extraction. High molecular weight genomic DNA was extracted as previously described (La Farina et al., 1993) from primary CRC and normal colon (as control) specimens.

DNA amplification. Mutations within the *p53* gene were detected by SSCP analysis following PCR amplification of the exons 5–8, performed as described previously (Russo et al., 1998). In every instance, negative (DNA was replaced with water) controls were amplified by PCR and included in the experiment. In all PCR assays, aerosol-resistant pipette tips were used to avoid cross-contamination (Eppendorf, Egham, Germany). The quality and the concentration of the amplification products were verified by 1.5% agarose gel electrophoresis and ethidium bromide staining.

SSCP analysis. About 100 ng aliquots of the amplified DNA fragments, purified and concentrated by filtration through Microcon 50 columns (Amicon, Beverly, MA), were denatured (Yap and Mc Gee, 1992) and analyzed by SSCP (Russo et al., 1998). In order to keep the temperature constant, the electrophoretic run was performed in a DGGE-2000 System (C.B.S. Scientific Company, Del Mar, CA) equipped with a KR-50A immersion chiller (PolyScience, Niles, IL). After the run, the gel was stained for 20 min with 0.5 μg/ml ethidium bromide in TBE and destained for 5 min; the DNA fragments were visualized under UV light. PCR-SSCP analysis was repeated twice for each sample to minimize the possibility of artifacts due to contamination or polymerase errors. Interpretation of SSCP analysis of DNA fragments was performed by consensus of two investigators. DNA of normal colon tissue from each patient was also amplified and run in parallel with matched tumoral DNA samples on SSCP gels, to evaluate the occurrence of no-somatic mutations or polymorphisms.

DNA reamplification and sequencing. Individual ssDNA fragments with shifted mobilities, compared to normal control, were electroeluted from polyacrylamide gel, reamplified, and sequenced as described previously (Albanese et al., 1997).

p53 immunohistochemistry

p53 immunostaining was assessed on 5 μm thick sections cut from formalin-fixed, paraffin-embedded tissue specimens. After the deparaffinization, sections were pre-treated with 3% H_2O_2, to quench endogenous peroxidase activity. Antigene retrival was performed by microwave eating in 10 mmol citrate buffer (pH 6.0). The sections were then immunostained with the DO-7 monoclonal antibody (dilution 1:60, Dako, Glostrup, Denmark). This antibody reacts with an epitope between amonoacids 19 and 26, recognizing both wild type and mutant forms of the p53 protein. After incubation with the biotinylated anti-mouse IgG secondary antibody, immunohistochemical reaction was performed by a standard peroxidase-labeled streptavidin-biotin procedure (LSAB+, Dako). The detection was performed using the AEC Substrate-Chromogen (Dako-AEC), the

TABLE 1. Patient characteristics (n = 160)

	No. of patients		No. of patients
Site		**Tumor grade**	
Proximal tumor	31	Well differentiated (G1)	23
Distal tumor	129	Mod differentiated (G2)	104
		Poorly differentiated (G3)	33
Tumor size (cm)		**Tumor type**	
≤ 5	60	Adenocarcinoma NOS	137
> 5	100	Mucinous	23
Dukes' stage		**Lymphohemative invasion**	
A	40	None	45
B	51	Present	115
C	41		
D	28		
Node status		**Lymphocytic infiltrate**	
Negative	101	Prominent	48
Positive	59	Non-prominent	112
Tumor growth		**Surgery**	
Expansive	20	Curative resection	137
Infiltrative	140	Non-curative resection	23

slides were then counterstained with Meyer hematoxylin/eosin and mounted with a permanent medium. Normal human serum was substituted for primary antibody on some sections, to serve as non-immune controls, while positive controls were sections of CRCs defined as strongly positive. Positive tumor cells were quantified by the pathologists (R.M.T. and V.M.) by evaluating at least 5000 cells from four different specimens of the same tumor and were expressed as the percentage ratio of the total number of tumor cells (Tomasino et al., 1994). A section was scored as positive when at least 5% of tumor cells showed staining.

All samples were evaluated blind, with no knowledge of either the biomolecular or the clinical pathological variables of patients. The tumors were divided unequivocally into two groups, negative and positive, on the basis of DO-7 immunohistochemistry.

Flow-cytometric analysis

DNA flow cytometry was performed on mechanically disaggregated aliquots of frozen tumor tissue as previously described (Russo et al., 1994). A laser flow cytometer (Ortho Diagnostic Systems K.K.; Matusaki Tateisi Electronics Co., Japan) was used for data acquisition. DNA-ploidy, DNA index, and SPF were determined as previously reported (Russo et al., 1994).

Statistical analysis

Fisher's exact test (StatXact Turbo, Cytel Software Corporation, Cambrige, MA) was used to evaluate the associations between biological and clinicopatological variables. P-values of less than 0.05 were considered significant (Cox, 1972).

RESULTS
Mutational analysis of *p53* gene

PCR-SSCP analysis was performed twice on genomic DNA from primary CRCs of 160 patients to assess the mutational status of exons 5–8 of the *p53* gene. DNA bands with abnormal electrophoretic mobility were detected by SSCP in 43% (68/160) of the cases (Fig. 2).
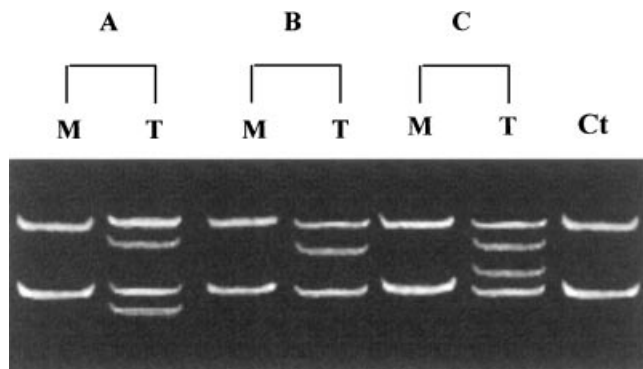
Fig. 2. SSCP analyses of exon 8 of the *p53* gene, amplified from CRC and mucosa genomic DNA of three patients (**A**,**B**,**C**). In each pair of lanes, the normal tissue DNA is at the left and the tumor DNA is at the right. The extra bands visualized in lanes 2, 4, and 6 correspond to ssDNA molecules harbouring mutations in codon 272 (GTG to ATG), 273 (CGT to CAT), and 282 (CGG to TGG), as confirmed by sequencing. Lane 7 shows negative control-DNA wild type.

The corresponding DNA fragments, after elution from the gel, were then sequenced to establish the exact site and nature of the genetic alteration (Table 2); overall 84 p53 mutations were identified and sequence data were obtained for 81 of them (for three cases DNA was no longer available). The distribution of the 84 mutations was as follows: 16 (19%) were in exon 5, 27 (32%) in exon 6, 30 (36%) in exon 7, and 11 (13%) in exon 8. Fourteen tumor samples were found to harbor two (nine in the same exon and three in two different exons) or three (two in two different exons) different p53 mutations. Forty-eight of the 84 mutations (57%) occurred in highly conserved domains (areas II–V) (the three cases found to be mutated by SSCP, which we were unable to sequence, were considered as mutated outside conserved regions, since they were amplified from specific and known genic regions.).

Accordingly, tumors with p53 mutations were classified into two groups: 56% of the cases (38/68) with at least one mutation in conserved areas of the *p53* gene (conserved); 44% (30/68 cases) with mutations outside the conserved areas (non-conserved). Nineteen of the 81 mutations were found to be frameshifts (23%) whereas 62 were single-nucleotide substitutions (77%). Of the latter, 45 were missense and six were nonsense mutations representing together 82%, while silent mutations were found in 11 cases (nine of them in codon 213, a previously identified site of polymorphism). Of these 11 cases, three presented also a second or third mutation (missense or frameshift) while eight had only the silent mutation. As expected, transitions (81%, 50 of 62) were much more frequent than transversions (19%), G:C to A:T mutations (50% at CpG sites) being the most represented (76%). In every case, the change was somatic since no mutations were found in the matched normal colic mucosa. The cases were also classified according to the specific domains of p53 affected by the mutations as follows: 18/65 cases (28%) with mutations of the L3, 11/65 cases (17%) with mutations of the LSH motif, and 36/65 cases (55%) with mutations outside L3 and LSH (of which six in L2). Silent mutations have been included in the wild-type group for statistical analysis since they do not determine any aminoacid change in the protein.

Immunohistochemical analysis

Forty-eight percent of the cases analyzed (77/160) presented positive staining for p53 (Fig. 3). Of these, 87% (67/77) presented exclusively positive nuclear staining, 9% (7/77) showed positive nuclear staining together with staining of the cytoplasm, and 4% (3/77) showed staining of the cytoplasm only.

Flow cytometric analysis

Flow cytometry was performed to obtain adequate DNA histograms for all normal and tumoral tissues. The coefficients of variation of the DNA-diploid peak ranged from 2.5 to 4.8% (median 3.4%). DNA-aneuploidy was found in 120/160 cases (75%), while 18% of these (22/120) showed multiclonality. The SPF ranged from 2.1 to 32.6% (median 18.3% and interquartile range 14.1–21.7%). The median SPF of DNA-aneuploid tumors was 19.2% while that of the DNA-diploid tumors was 12.4% ($P < 0.01$). By using the SPF median value as cut-off

TABLE 2. Localization and type of mutations, immunohistochemestry expression of p53 in 68 patients with CRC

Type mutation		Mutated exon	Mutated codon	Nucleotide change	Aminoacid change	Type of mutation	CD	Zn-BD	IHC
Single mutations									
Missense	•	5	141	tgc→tac	cys→tyr		Y		+
	•	5	141	tgc→tac	cys→tyr		Y		+C
	•	5	141	tgc→tcc	cys→ser		Y		−
	•	5	151	ccc→acc	pro→thr		N		+
	•	5	151	ccc→tcc	pro→ser		N		−
	•	5	158	cgc→cac	arg→his		N		+
	•	5	158	cgc→cac	arg→his		N		−
	•	5	163	tac→ttc	tyr→phe		N	L2	−
	•	5	174	agg→aag	arg→lys		Y	L2	+
	•	5	174	agg→aag	arg→lys		Y	L2	−
	•	6	195	atc→ttc	ile→phe		N	L2	+
	•	6	201	ttg→ttc	leu→phe		N		−
	•	6	220	tat→tgt	tyr→cys		N		+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	7	248	cgg→tgg	arg→trp		Y	L3	+
	•	8	272	gtg→atg	val→met		Y	LSH	+C
	•	8	272	gtg→atg	val→met		Y	LSH	+
	•	8	273	cgt→cat	arg→his		Y	LSH	+
	•	8	273	cgt→cat	arg→his		Y	LSH	+
	•	8	273	cgt→cat	arg→his		Y	LSH	+
	•	8	273	cgt→cat	arg→his		Y	LSH	+
	•	8	273	cgt→cat	arg→his		Y	LSH	+
	•	8	278	cct→tct	pro→ser		Y	LSH	+
	•	8	278	cct→tct	pro→ser		Y	LSH	−
	•	8	282	cgg→tgg	arg→trp		Y	LSH	+
Nonsense	•	6	192	cag→tag	gln→STOP		N	L2	−
	•	6	213	cga→tga	arg→STOP		N		+
	•	6	213	cga→tga	arg→STOP		N		−
Frameshift	•	5	152	ccg→cccc	pro→		N		−
	•	5	155	acc→ac-	thr→		N		−
	•	5	177	ccc→cc-	pro→		Y	L2	−
	•	6	196	cga→ctga	arg→		N		−
	•	6	202	cgt→cgct	arg→		N		−
	•	6	206/	ttg→tt-/	leu/→		N		−
		6	/207	/gat→at	/asp→				
	•	6	206/	ttg→tt-/	leu/→		N		−
		6	/207	/gat→at	/asp→				
	•	6	214	cat→caat	ist→		N		+
	•	7	255	atc/aca→	ile/thr→		Y		−
		7	/256	at-/−a	ile/-				
Silent	•	5	152	ccg→cct	pro→pro		N		+
	•	6	213	cga→cgg	arg→arg		N		+
	•	6	213	cga→cgg	arg→arg		N		+
	•	6	213	cga→cgg	arg→arg		N		−
	•	6	213	cga→cgg	arg→arg		N		−
	•	6	213	cga→cgg	arg→arg		N		−
	•	6	213	cga→cgg	arg→arg		N		−
	•	6	213	cga→cgg	arg→arg		N		−
Double mutations	•	5	141	tgc→cgc	cys→arg	Missense	Y		+C
		6	204	gag→tag	glu→STOP	Nonsense	N		
	•	6	193	cat→cag	his→gln	Missense	N	L3	+C
		7	242	tgc→tac	cys→tyr	Missense	Y		
	•	6	204	gag→tag	glu→STOP	Nonsense	N		−
		7	206	ttg→tag	leu→STOP	Nonsense	N		
	•	6	213	cga→cgg	arg→arg	Silent	N		+
		8	282	cgg→tgg	arg→trp	Missense	Y	LSH	
	•	7	238	tgt→tat	cys→tyr	Missense	Y	L3	+
		7	249	agg→ag-	arg→	Frameshift	Y		
	•	7	244	ggc→agc	gly→ser	Missense	Y	L3	+
		7	249	agg→ag-	arg→	Frameshift	Y		
	•	7	244	ggc→agc	gly→ser	Missense	Y	L3	+C
		7	249	agg→ag-	arg→	Frameshift	Y		
	•	7	244	ggc→agc	gly→ser	Missense	Y	L3	−
		7	249	agg→ag-	arg→	Frameshift	Y		
	•	7	244	ggc→agc	gly→ser	Missense	Y	L3	−

TABLE 2. (*Continued*)

Type mutation		Mutated exon	Mutated codon	Nucleotide change	Aminoacid change	Type of mutation	CD	Zn-BD	IHC
		7	249	agg→ag-	arg→	Frameshift	Y		
	●	7	244	ggc→agc	gly→ser	Missense	Y	L3	−
		7	249	agg→ag-	arg→	Frameshift	Y		
	●	7	244	ggc→gcc	gly→ala	Missense	Y	L3	−
		7	249	agg→cgg	arg→arg	Silent	Y		
	●	7	253	acc→ac-	thr→	Frameshift	Y		−
		7	255	atc→a-c	ile→	Frameshift	Y		
Triple mutations	●	6	213	cga→cgg	arg→arg	Silent	N	L3	+
		7	248	cgg→cag	arg→gln	Missense	Y		
		7	249	agg→ag-	arg→	Frameshift	Y		
	●	6	220	tat→tgt	tyr→cys	Missense	N	L3	−
		7	244	ggc→agc	gly→ser	Missense	Y		
		7	249	agg→ag-	arg→	Frameshift	Y		
Not sequenced	●	5					N		+
	●	6					N		+
	●	6					N		−

●, Single case; +C, Positive cytoplasmatic immunostaining.

point, tumors were accordingly divided into low ($\leq 18.3\%$) and high ($> 18.3\%$) SPF tumors.

p53 mutational status versus immunohistochemistry (IHC)

There was 57% (91/160) agreement between expression and *p53* genetic status, with 38 cases (24%) positive for both and 53 cases (33%) negative for both. Of the other cases, 39 (24%) were positive at IHC, but negative at mutational analysis, while 30 (19%) were IHC-negative cases and positive at mutational analysis (Table 3).

Relationship between *p53* gene status, biological and clinicopathological data

Overexpression of p53 proved to be associated with mutations in the conserved areas ($P < 0.01$), with those in the L3 domain of the gene ($P < 0.01$) and with the type of mutation (missense vs. frameshift and transitions vs. transversions vs. frameshift) ($P < 0.01$) (Table 4). Furthermore, the overexpression of p53 was associated with DNA-aneuploid multiclonal tumors ($P < 0.05$) and with distal site ($P < 0.05$) (Table 5); mutations in the conserved areas of the gene were associated with DNA-aneuploid tumors ($P < 0.05$), with high proliferative activity ($P < 0.01$) and with distal site ($P < 0.05$) while mutations in the L3 domain proved to be associated with high proliferative activity ($P < 0.05$) (Table 6). No significant relationship was seen between the presence (any mutations) or type of p53 mutations and the clinicopathological variables analyzed. DNA-aneuploidy was associated with distal tumors ($P < 0.01$), histological grade (G3) ($P < 0.05$), advanced Dukes' stage (C and D) ($P < 0.01$), lymph node metastases ($P < 0.01$), and high SPF ($> 18.3\%$) ($P < 0.01$) (data not shown).

DISCUSSION

Genetic alterations of the oncosuppressor *p53* gene are frequently found in many types of neoplasms (Hollstein et al., 1991; Lane, 1994). Mutations in this gene are usually screened either by direct analysis of its nucleotide sequences, after a preliminary discrimination between wild type and mutant molecules by any of the many available procedures, all of which are however less than 100% sensitive, or by immunohistochemistry, to detect accumulation of the protein within the cell. This is based on the principle that p53, normally expressed at very low levels due to the rapid turnover caused by a negative autoregulatory feedback loop operating through MDM2, may be overexpressed when mutated (Lane and Hall, 1997). This may be due to several events: mutations affecting the central, core domain of p53 may reduce or even abolish its DNA binding activity; in such cases, p53 might no longer be able to efficiently activate the transcription of its target genes, and in particular of MDM2. As a consequence, p53 itself would no longer be ubiquitinated, exported to the cytoplasm, and destroyed. Mutations affecting the N-terminal coding region of p53 might also lead to its stabilization if they alter the site of interaction with MDM2 and/or the transactivation domain, which are both located in this portion of the gene. In the latter case, the mutated p53 would again be unable to upregulate MDM2 expression, while in the first would no longer be able to bind to it.

However, it is still not clear whether all possible p53 mutations lead to increased cellular levels of the protein, nor whether IHC staining for p53 is always a hallmark of mutations in the protein.

Among the 160 cases of CRC analyzed in the present report, *p53* gene mutations were found, by PCR-SSCP analysis, in 68 (42%), which is well within the range (23–61%) indicated by other authors (Kikuchi-Yanoshita et al., 1992; Dix et al., 1994; Borresen-Dale et al., 1998; Kressner et al., 1999). Fifty-six percent of mutations observed in our series were found in four of the five highly conserved areas of the gene, as reported by other authors (Goh et al., 1995, Jernvall et al., 1997; Kressner et al., 1999). These areas include important regions for p53 interaction with DNA, in particular the L3 zinc-binding domain and the LSH motif (see Fig. 1). Similar to the results reported by (Borresen-Dale et al., 1998), in our own series 22% (18/81) of the mutations occurred in L3 and 14% (11/81) in LSH. Our data confirm that arginines 248 and 273, aminoacids within these domains interacting directly with DNA, are among the most frequently mutated residues (11 and 6%, respectively).
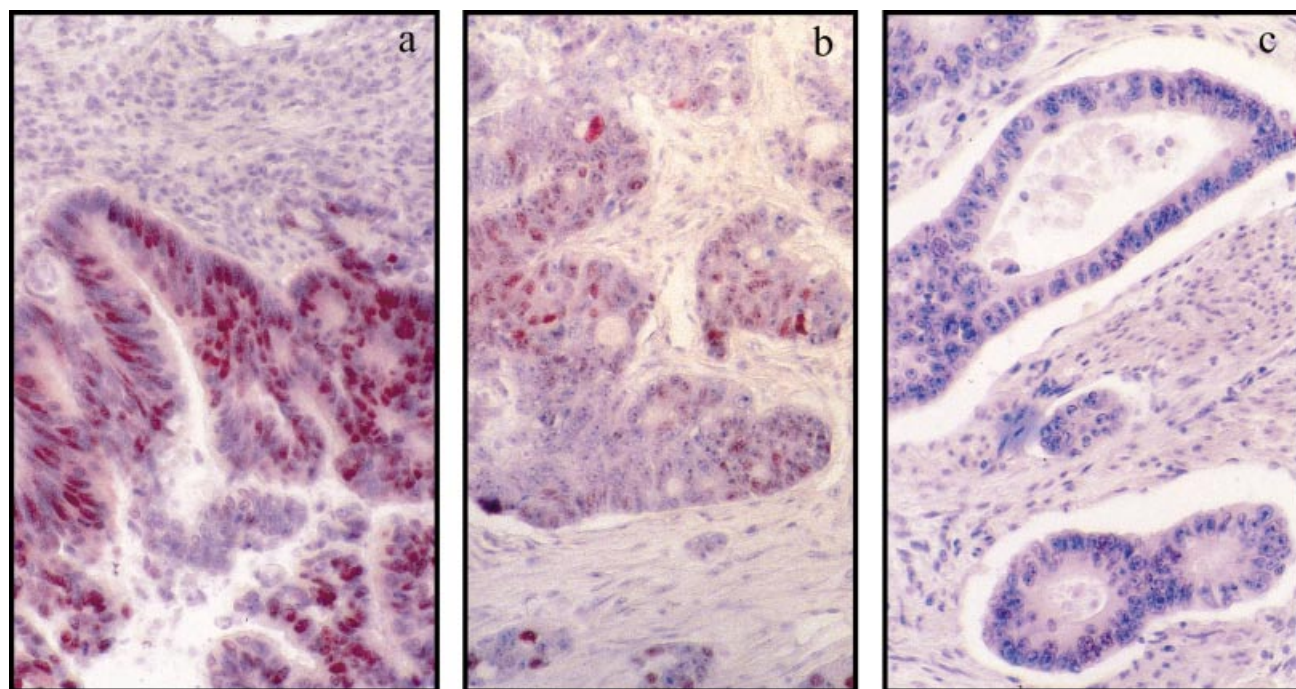
Fig. 3. Strong nuclear p53 expression in many glandular cells of a moderately differentiated colonic carcinoma is shown at × 220 magnification (**a**); some scattered glandular cells of a moderately differentiated colonic carcinoma show a weak p53 nuclear expression (× 220) (**b**); negative control (× 250) (**c**).

The amino acids localized in L2, needed for the folding and stabilization of the central domain (Cho et al., 1994), when mutated, are also responsible for the loss of p53 DNA-binding capacity. Mutations in this area were observed less frequently in our own series (7%).

In our study, p53 overexpression was detected in 48% of the cases (77/160). This value is similar to that reported by several other authors, whose results range from 42 to 74% (Scott et al., 1991, Auvinen et al., 1994; Bouzourene et al., 2000; Kaserer et al., 2000; Jansson et al., 2001). The median cut-off point used in our study (5%) should reduce the possibility of assessing false positive reactions due to background or to artefacts.

Only 10 of the 160 cases (6%) which we analyzed presented positive staining in the cytoplasm. Literature reports cytoplasmic staining ranges of between 10 and 50% (Sun et al., 1992; Bosari et al., 1994; Sun et al., 1996). This variability may be due to the antibody used for the immunohistochemical analysis. In fact, the monoclonal antibody DO7 used in our study is more likely to give a positive nuclear result, compared with the polyclonal antibody CM1 used in several other studies (Sun et al., 1992; Bosari et al., 1994), which more frequently gives a positive cytoplasmic staining. The analysis of our data shows only a partial (57%) agreement between the level of p53 expression and its genetic status. The variability in the concordance values reported in the literature regarding the analysis of a large number of cases (at least 50), ranges from 53 to 70% (Dix et al., 1994; Veloso et al., 2000; Jansson et al., 2001). As other authors have suggested (Dix et al., 1994; Leahy et al., 1996; Adrover et al., 1999), there may be several explanations for those cases in which p53 appears unaltered by SSCP but is positive at IHC, as observed in 24% of the tumors in our own study. An accumulation of the non-mutated protein may be due to the formation of complexes between p53 and other cellular proteins (MDM2, heat-shock proteins), or viral proteins (SV40 antigen, E1b adenovirus, E6), or to alterations in any of several factors which control p53 stability. Alternatively, a p53 mutation may indeed be present, but not detected either because it does not lead to an altered electrophoretic mobility of the corresponding DNA fragment, at least under the conditions utilized for the analysis, or because it is localized outside the analyzed region (exons 5–8), as reported to occur for 13–20% of all mutations (Greenblatt et al., 1994, Hartmann et al., 1995). p53 mutations in the N-terminal region, in particular, may lead to increased stability and accumulation of p53 if they prevent the interaction with MDM2, whose minimum binding site on p53 is mapped between

TABLE 3. Agreement between over-expression (analyzed by IHC) and the presence of p53 mutations (analyzed by PCR/SSCP)

	p53 Mutational status		
	SSCP (−)	SSCP (+)	Total
p53 Expression			
IHC (−)	53/160 (33%)	30/160 (19%)	83/160 (52%)
IHC (+)	39/160 (24%)	38/160 (24%)	77/160 (48%)
Total	92/160 (57.5%)	68/160 (42.5%)	

IHC, immunohistochemistry; SSCP, single strand conformation polymorphism.

TABLE 4. Relationships between p53 expression and p53 mutational status in 160 patients with CRC

	p53 Expression		
	Negative	Positive	P
p53 Mutational status			
Wild type*	58	42	
Non-conserved areas	13	9	
Conserved areas	12	26	< 0.01
Wild type*	58	42	
Outside LSH/L3	18	10	
LSH	1	10	
L3 (3 mutations not characterized)	5	13	< 0.01
Wild type*	58	42	
Missense	7	27	< 0.01
Nonsense	4	1	
Frameshift	13	5	
Wild type*	58	42	
Transition	6	24	< 0.01
Transvertion	5	4	
Frameshift	13	5	

*Silent mutations have been included in the wild-type group.

residues 18 and 23 (Kussie et al., 1996). These amino-acids are outside the exons analyzed in this study, so at least some of the cases positive for immunohistochemistry and negative for p53 mutations may be explained by mutations occurring in these sites. Several explanations might also be proposed for the finding of *p53* gene mutations in specimens which are negative at the IHC analysis (Dix et al., 1994, Leahy et al., 1996, Adrover et al., 1999). Thus, for example, the antibody used for the immunohistochemical analysis may be unable to identify the mutated protein, even though overexpressed, because of structural changes in some of its epitopes. Alternatively, the specific mutation of p53 found in the specimen may not lead to its accumulation. In our own study, 30 cases negative at IHC staining presented mutations and some even two or three independent mutations (for a total of 38) in the *p53* gene (in one case, no sequence data were available). Eighteen of these mutations (for a total of 16 cases) (Table 2), were either single-nucleotide substitutions which resulted in a stop codon or small insertions or deletions which caused a frameshift, often giving rise to a stop codon downstream. These mutations, which could lead to the production of a truncated protein, or to a protein with a C-terminal aminoacid sequence very different from that of the wild

TABLE 5. Significant relationships between p53 expression, site, and DNA ploidy

	p53 Expression		
	Negative	Positive	P
Site			
Proximal tumors	21	10	
Distal tumors	62	67	< 0.05
DNA-ploidy			
Diploid	29	11	
Aneuploid monoclonal	44	54	
Aneuploid multiclonal	10	12	< 0.05
Total	83	77	

type, may not cause its stabilization and consequent accumulation. Alternatively, the mutations giving rise to a premature termination codon could result in a rapid degradation of the mutated mRNA (Mendell and Dietz, 2001). Five additional mutations found in IHC negative tumors were silent. This type of mutations does not alter either the structure or the function of p53, and therefore has no effect on the normal control of its stability. Thus, in at least 17 of the SSCP-positive, IHC-negative cases the absence of staining for p53 is not surprising, because they present only silent, nonsense or frameshift mutations. In 12 different specimens, however, we found 13 missense mutations, at least some of which should alter the DNA-binding and transcription-activating function of p53. The lack of p53 overexpression in these cases is more intriguing, also in the light of the fact that other specimens with identical p53 mutation were IHC-positive. One possible explanation of these findings is that, due to a different sensitivity of the PCR-SSCP and IHC methods of analysis, gene mutations can be detected even in specimens in which they affect a percentage of cells too low to give a positive staining result. Alternatively, in some cases the observed results may be due to additional mutations, perhaps in genes coding for other factors modulating the level of p53 expression. A similar consideration may apply to those few IHC-positive cases in which we detect silent, nonsense, or frameshift mutations not expected to lead to protein accumulation.

Statistical analysis of our data revealed that although overall mutations of p53 are not associated with the overexpression of the protein, the subset of mutations specifically affecting conserved areas of the gene or domains of particular importance for the function of the protein (loop L3) and specific types of mutation (missense and transitions) do indeed show a significant association with increased expression ($P < 0.01$), evidence that not all p53 mutations have the same value with regard to protein half-life.

An assessment of the possible associations between p53 alterations and the traditional clinicopathological variables further showed that the occurrence of particular gene alterations depended on the tumor site. In fact, both overexpression of p53 and mutations in the conserved areas of the gene were more frequent in distal carcinomas of the colon (respectively $P < 0.05$, $P < 0.05$). Some other authors have confirmed our results revealing that overexpression of p53 and certain p53 mutations, in the conserved areas or in specific exons of the gene (Jernvall et al., 1997; Diez et al., 2000), were significantly higher in distal than in proximal CRC. These data would seem to support the observations made by Weisburger (1991), that proximal and distal tumors involve different types of epidemiological behavior, and by (Beart et al., 1983) regarding the existence of two different pathways of tumoral progression of CRCs originating in the two tracts.

Moreover, our results indicated that p53 mutations in the conserved regions are significantly related to flow-cytometry parameters, and that the overexpression of p53 is associated with DNA-aneuploidy ($P < 0.05$), while p53 mutations in the L3 domain are associated with high SPF. The first of these observations, i.e., that mutations of the gene proved to be significantly associated with

TABLE 6. Significant relationships between p53 mutational status, site, SPF, and DNA-ploidy

	p53 Mutational status			
	Wt*	Non-conserved areas	Conserved areas	P
Site				
Proximal tumors	26	3	2	
Distal tumors	74	19	36	< 0.05
SPF				
≤ 18.3%	60	10	11	
> 18.3%	40	12	27	< 0.01
DNA-ploidy				
Diploid	35	2	3	
Aneuploid	65	20	35	< 0.05
Total	100	22	38	

	p53 Mutational status				
	Wt*	Outside LSH/L3	LSH	L3	P
SPF					
≤ 18.3%	60	12	5	4	
> 18.3%	40	16	6	14	< 0.05
DNA-ploidy					
Diploid	35	4	0	1	
Aneuploid	65	24	11	17	< 0.05
Total	100	28	11	18	

*Silent mutations have been included in the wild-type group.

DNA aneuploidy and high SPF, might be due to the fact that high proliferative activity leads to an increase in the likelihood of accumulating DNA damages and the formation of aneuploid clones. However, the association between positive staining of p53 and DNA-aneuploidy but not high SPF, and the association between mutations in L3 and high SPF but not DNA-aneuploidy, may indicate that p53 control the genetic stability and the proliferation rate of the cell through different and, at least in part, independent pathways.

In conclusion, IHC and mutational analysis of p53 thus appear to be complementary rather than equivalent methods of investigation to assess the occurrence of alterations in this key regulator of cell proliferation, genetic stability, and apoptosis. Only in some cases do they, in fact, provide overlapping information; in addition, IHC may reveal perturbations of molecular mechanisms involved in the control of p53 expression, intracellular localization, and stability not dependent on the status of the p53 protein itself, while only sequence analysis of the *p53* gene can discover mutations (e.g., nonsense, frameshift) which lead to the absence of a functional protein without concomitant accumulation.

Therefore, the combination of the mutational status and immunohistochemistry of p53, and flow cytometric data may provide an important insight into the biological features of CRCs.

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